Original Article

Advanced Rai Stage in Patients with Chronic Lymphocytic Leukaemia Correlates with Simultaneous Hypermethylation of Plural Tumour Suppressor Genes

(chronic lymphocytic leukaemia / aberrant hypermethylation / tumour suppressor genes / Rai stage / methylation-specific polymerase chain reaction)

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Abstract. Hypermethylation of CpG islands within gene promoters is one of various mechanisms of gene silencing involved in the pathogenesis of human cancer. By using methylation-specific polymerase chain reaction we explored aberrant promoter methylation of five tumour suppressor genes in 29 patients with chronic lymphocytic leukaemia. Aberrant methylation of *DLC1*, *SHP1*, *p15* and *p16* occurred, respectively, in 89.7 %, 70 %, 62.1 % and 31 % of patients at diagnosis. *Lamin A/C* was unmethylated in all the samples. Hypermethylation of at least one gene was detected in 96.6 % of patients. Concurrent methylation of two or more genes correlated with Rai stage at diagnosis.

Introduction

Chronic lymphocytic leukaemia (CLL) is a common B-cell lymphoproliferative disorder characterized by variable clinical course. The extent of the disease in an individual is assessed by the staging system proposed by Rai et al. (1975) and Binet et al. (1981). In addition, a number of biological prognostic factors have been described to discriminate between indolent and aggressive forms of CLL. These include cytogenetic aberrations (Dohner et al., 1997, 2000), immunoglobulin heavy-chain variable region (*IgVH*) gene mutational status, expression of CD38 (Damle et al., 1999; Hamblin et al., 1999, 2002; Thunberg et al., 2001; Krober et al., 2002; Oscier et al., 2002; Stilgenbauer et al., 2002; Guarini et al., 2003; Vasconcelos et al., 2003) and ZAP70 (Rosenwald et al., 2001; Crespo et al., 2003; Rassenti et al., 2004) proteins and expression of microRNAs (Calin et al., 2004; Chen and Lodish, 2005).

DNA methylation, catalysed by DNA methyltransferases, involves the addition of a methyl group to the carbon-5 position of the cytosine ring in a CpG dinucleotide to become methylcytosine (Singal and Ginder, 1999; Baylin and Herman, 2000; Robertson and Wolffe, 2000; Chim et al., 2002). CpG dinucleotides are either scattered throughout the genome, or are found in stretches of CpG-rich DNA, referred to as CpG islands. CpG islands are mainly located at gene promoters where they are protected from methylation, so that these genes are in a transcription-ready state. On the other hand, non-promoter CpG dinucleotides are found in repeat regions and are often methylated. Hypermethylation of CpG islands of the promoter region genes is associated with transcriptional inactivation and it is one of the mechanisms of tumour suppressor gene (TSG) inactivation (Herman and Baylin, 2003). Hypermethylation of CpG islands has been described in various tumour types, including CLL (Rush et al., 2004; Seeliger et al., 2009). Tumour suppressor genes may be inactivated by methylation, which may confer a growth advantage contributing to leukaemogenesis. The genes interesting from this point of view could be represented by two widely studied TSGs *p15* and *p16*, lamin A/C, *DLC1* and *SHP1*. It is well established that methylation of the promoters of these genes correlates with their reduced expression

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Abbreviations: CLL – chronic lymphocytic leukaemia, IgVH – immunoglobulin heavy-chain variable region, MSP – methylation-specific polymerase chain reaction, NHL – non-Hodgkin lymphoma, PCR – polymerase chain reaction, TSG – tumour suppressor gene.

(Foster et al., 1998; Oka et al., 2002; Wong et al., 2003; Agrelo et al., 2005).

p15 and *p16* encode cyclin-dependent kinase inhibitors important for G1 cell cycle arrest (Hirama and Koeffler, 1995; Koh et al., 1995). A-type lamins are important in maintaining the stability of nuclear lamina, and they also have a central role in maintaining the function of transcription factors required for the differentiation of adult stem cells (Hutchison and Worman, 2004). Mutations in the lamin A/C gene have been shown to cause several tissue-specific inherited diseases, such as Emery-Dreifuss muscular dystrophy or Dunnigan-type familial partial lipodystrophy (Broers et al., 2004). Its hypermethylation was detected in patients with diffuse large B-cell lymphoma (Agrelo et al., 2005). *DLC1* (deleted in liver cancer) is considered as a potential tumour suppressor, its hypermethylation was detected in several leukaemia and lymphoma types (Shi et al., 2007; Ying et al., 2007; Pike et al., 2008). *SHP1* is expressed primarily in haematopoietic cells (Oka et al., 2002). SHP1 acts as a growth inhibitor in B cells by down-regulating the intracellular effects of immunoglobulin binding, thus requiring multiple receptor binding to initiate B-cell activation and proliferation (Cyster and Goodnow, 1995; Pani et al., 1995). B lymphocytes with decreased SHP1 activity are thus more likely to proliferate and escape apoptosis (Cyster and Goodnow, 1995; Pani et al., 1995). *SHP1* hypermethylation was described in several haematologic malignancies (Koyama et al., 2003; Chim et al., 2004; Reddy et al., 2005; Amara et al., 2007, 2008; Chim et al., 2007).

In this study, we investigated the frequency of methylation of the above-mentioned genes showing the *DLC1* gene to be extensively methylated in CLL. The clinicopathological and prognostic impacts of aberrant gene methylation in CLL were also examined. We have found that patients with advanced Rai stage at diagnosis usually carry several methylated genes concurrently.

Material and Methods

Patients

Twenty-nine patients (20 males and 9 females, median age 55.7, range 44–69 years) with CLL were included into the analysis. All the patients enrolled in this study had immunophenotypically defined B-CLL as outlined by the modified 1996 NCI criteria (Cheson et al., 1996). The clinical stage was evaluated according to Rai et al. (1975). There were three (10.3 %) stage 0, twelve (41.4 %) stage I, eight (27.6 %) stage II, five (17.2 %) stage III and one (3.5 %) stage IV patients by Rai staging system. Of 23 patients with a known mutational status, 22 had unmutated *IgVH* genes. This bias in the mutational status was due to primary selection of patients who needed treatment with immunochemotherapy.

Methylation-specific polymerase chain reaction (MSP)

DNA was extracted from samples using the salting-out method (Miller et al., 1988). DNA was modified by bisulphite reaction using the EpiTect DNA Modification kit (Qiagen, Hilden, Germany). After completion of the reaction, all unmethylated cytosines were deaminated and converted to uracil, while methylated cytosines remained unchanged. Primer sequences for the methylated and unmethylated alleles were as previously published for *p15*, *p16* (Herman et al., 1996), *SHP1* (Oka et al., 2002), *DLC1* (Wong et al., 2003) and lamin A/C (Agrelo et al., 2005) (Table 1). The polymerase chain reaction (PCR) mixture contained 2 µl of bisulphite-treated DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 300 nM of each primer, 1x PCR buffer II and 1.5 units of AmpliTaq Gold (PE Biosystems, Foster City, CA) in a final volume of 30 µl. PCR conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, annealing temperature (specified for each primer pair in Table 1) for 30 s, 72 °C for 90 s, and finally 5 min at 72 °C. PCR products were separated in 10 % non-denaturing polyacrylamide gel and visualized by ethidium bromide staining. All tests were performed in duplicate. Each MSP reaction contained a positive control with methylated DNA, a negative control with DNA from 30 normal donors and reagent blanks. The sensitivity of MSP was estimated by serial 10-fold dilution of methylated DNA in normal donor DNA, followed by bisulphite modification and amplification by MSP, and was found to be 1×10^{-3} for *DLC1*, *p16* and *SHP1*, and 1×10^{-4} for *p15*.

Table 1. Sequences of PCR primers. M-methylated, U-unmethylated

Gene	Forward primer	Reverse primer	Ann. t., °C
p15-M	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	60
p15-U	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	60
p16-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65
p16-U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	60
SHP-1-M	GAACGTTATTATAGTATAGCGTTC	TCACGCATACGAACCCAAACG	60
SHP-1-U	GTG AAT GTT ATT ATA GTA TAG TGT TTG G	TTC ACA CAT ACA AAC CCA AAC AAT	59
DLC-1-M	TTT AAA GAT CGA AAC GAG GGA GCG	CCC AAC GAA AAA ACC CGA CTA ACG	55
DLC-1-U	TTT TTT AAA GAT TGA AAT GAG GGA GTG	AAA CCC AAC AAA AAA ACC CAA CTA ACA	58
lamin-M	TTA TTA GAG TTT TTG TTT CGG CGT C	CGC CGA CCG ACT AAC TCT CG	60
lamin-U	AGG ATT TAT TAG AGT TTT TGT TTT GGT GTT	CAA AAT ACA CCA ACC AAC TAA CTC TCA	60

Ann. t. – annealing temperature

Statistical analysis

The Spearman ρ test was performed to statistically evaluate the correlations between the methylation level of individual genes and their combinations and the patients' clinical characteristics such as clinical stage at diagnosis, age, sex, and CD38 expression. We used the log-rank test of Kaplan-Meier to determine the association between hypermethylation of individual genes and overall survival. $P < 0.05$ was considered statistically significant.

Results

MSP in primary CLL marrow samples

We determined CpG island methylation at five loci in bone marrow samples from 29 patients with chronic lymphocytic leukaemia. Thirty normal peripheral blood donors were tested and the results were all negative for methylation of all the genes studied.

The methylation patterns varied grossly in individual patients. Of the 29 marrow samples, 28 (96.6 %) showed promoter methylation of at least one gene, with a maximum of four methylated genes. Four samples showed methylation at one gene, six at two genes, fifteen at three genes, and three at four genes. A high proportion of samples (89.7 %) were methylated at the *DLC1* (Fig. 1) locus, whereas 70 %, 62.1 % and 31 % were methylated at the *SHP1*, *p15* and *p16* loci, respectively. In contrast, none of the patients showed methylation of the lamin A/C gene.

Correlation of clinicopathological characteristics and MSP

We compared molecular and clinicopathological features of CLL patients. We found a good correlation between methylation of *DLC1* and *p15* genes ($P = 0.0182$). In addition, there was a statistically significant correlation between Rai stage at diagnosis and simultaneous methylation of two or more genes.

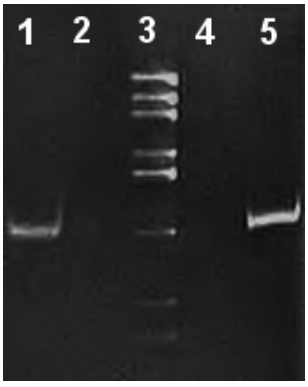

Fig. 1. M-MSP of the *DLC1* gene. Lane 1: positive sample, Lane 2: negative sample, Lane 3: marker, Lane 4: negative control from healthy donors, Lane 5: positive control (*in vitro* methylated DNA).

We used Spearman ρ test to analyse whether any of the methylated genes or their different combinations might predict patient's clinical stage of the disease. Our results showed that the potency to predict higher Rai stage at diagnosis is possessed by only two or better three simultaneously methylated genes. When considering simultaneous methylation of two genes, the most predictive combination of genes was *SHP1* and *p15* ($P = 0.0445$). In the case of simultaneous methylation of three genes, the most suitable was the hypermethylation pattern of *SHP1*, *p15* and *p16* ($P = 0.0165$). Moreover, when dichotomizing Rai stage into two groups, Rai 0, I versus Rai II-IV, an even better correlation was observed between dichotomic Rai stage and concurrent methylation of the couple of methylated genes *SHP1* and *p15* ($P = 0.0153$) and the triad of genes *p15*, *p16* and *SHP1* ($P = 0.0015$) (Table 2). The strongest correlation was found between dichotomic Rai stage and simultaneous methylation of at least any three genes ($P = 0.0004$), e.g. patients in higher Rai stage tended to have multiple methylated genes (Fig. 2).

No association was identified between the patterns of aberrant gene methylation and other clinicopathological features at presentation, including age, sex and CD38 expression. There was no significant impact of *DLC1*, *SHP1*, *p15* and *p16* methylation on the median overall survival of the 29 CLL patients studied (data not shown).

Discussion

We have studied the methylation profile of a panel of five tumour suppressor genes as well as the correlation of these aberrant methylations with clinicopathological characteristics in a pilot cohort of patients with CLL. We found that *DLC1*, *SHP1* and *p15* genes were fre-

Table 2. Statistical significance of the correlation between the dichotomized Rai stage and methylated genes. r – Spearman's coefficient

Methylated gene (combination of genes)	Statistical significance of Spearman's rank correlation coefficient	
	r	P value
SHP1	0.350	0.0629
DLC1	0.328	0.0822
p15	0.329	0.0818
p16	0.247	0.1967
SHP1+DLC1	0.424	0.0217
SHP1+p15	0.446	0.0153
SHP1+p16	0.429	0.0202
DLC1+p15	0.364	0.0519
DLC1+p16	0.385	0.0392
p15+p16	0.439	0.0172
SHP1+DLC1+p15	0.464	0.0113
SHP1+DLC1+p16	0.500	0.0058
SHP1+p15+p16	0.563	0.0015
DLC1+p15+p16	0.479	0.0086
SHP1+DLC1+p15+p16	0.568	0.0013
more than two genes	0.613	0.0004

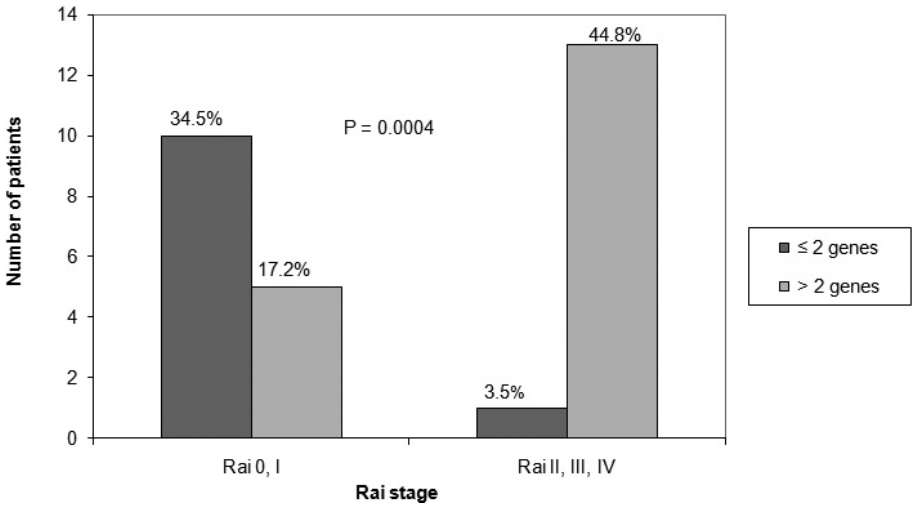

Fig. 2. Correlation between the dichotomized Rai stage at diagnosis and the number of methylated genes.

quently methylated in CLL, whereas *p16* was methylated with lower frequency and lamin A/C was unmethylated in all the patients. None of the normal control peripheral blood samples showed methylation of any of the genes tested.

The highest frequency of methylation was detected in the *DLC1* gene. It was identified as a candidate tumour suppressor and its expression was lost or downregulated in various cancers, including liver, breast, lung, brain, stomach, colon and prostate cancers due to either genomic deletion or aberrant DNA methylation (Yuan et al., 1998, 2003, 2004; Ng et al., 2000; Kim et al., 2003, 2007; Plaumann et al., 2003; Wong et al., 2003; Guan et al., 2006; Ullmannova and Popescu, 2006, Song et al., 2006; Seng et al., 2007). Its aberrant methylation was also detected in 60–90 % of various types of primary non-Hodgkin lymphomas (NHLs) (Shi et al., 2007; Ying et al., 2007). As yet, no data on *DLC1* methylation in CLL are available in the literature. We found this gene to be aberrantly hypermethylated in 89.7 % of CLL patients. The high frequency of *DLC1* methylation found in our study implied that it might play a role in leukaemogenesis.

SHP1 was another frequently methylated gene. It has been shown to be frequently silenced in leukaemias, lymphomas and multiple myeloma (Zhang et al., 2000; Oka et al., 2002), but no study has addressed *SHP1* methylation in CLL specifically. In our study, 70 % of patients had methylation of the *SHP1* promoter. This frequency was comparable with previous reports that demonstrated *SHP1* methylation in 94 % in leukaemias (Reddy et al., 2005) and in 75–100 % in NHLs (Koyama et al., 2003; Reddy et al., 2005).

p15 and *p16* are two closely linked tumour suppressor genes located at 9p21 (Kamb et al., 1994; Nobori et al., 1994). In human cancers, *p16* is frequently inactivated by homozygous deletion, point mutations or methylation of its promoter region (Cairns et al., 1995; Herman et al., 1995). *p15* is often homozygously co-deleted in solid tumours with homozygous deletion of *p16* (Chim et al., 2002). In haematologic malignancies, *p15* is frequently methylated in several leukaemias and rarely in lymphoma, while promoter methylation of *p16* is common in lymphoma but rarely seen in leukaemia (Herman et al., 1997). In CLL, methylation of both *p15* and *p16* has been detected with lower frequency than in our study. Previous studies found the *p15* promoter to be methylated in 12–35 % (Chim et al., 2006; Papageorgiou et al., 2007) of patients and methylation of *p16* has been found in less than 20 % of cases (Martel et al., 1997; Chim et al., 2006; Tsirigotis et al., 2006). In the present study, we found that 62 % and 31 % of CLL patients exhibit methylation of *p15* and *p16*, respectively.

The lamin A/C gene encodes the lamins A and C. It has been described that the expression of the A-type lamins is reduced or absent in subsets of cells with a low degree of differentiation and/or cells that are highly proliferating (Rober et al., 1989; Broers et al., 1997), including human malignancies (Hutchison and Worman, 2004), especially leukaemias and lymphomas (Stadelmann et al., 1990; Lin and Worman, 1997). Hypermethylation of lamin A/C was found to be associated with poor outcome in diffuse large B-cell lymphoma (Agrelo et al., 2005). However, none of CLL patients in our study showed methylation of this gene.

We have correlated aberrant methylation of the abovementioned genes with several clinicopathological features of patients, including age, sex, CD38 expression, Rai stage at diagnosis and overall survival. We found a correlation between methylation patterns of two genes, *p15* and *DLC1*. Moreover, we found that simultaneous methylation of at least two genes correlated with Rai stage at diagnosis. In addition, we identified optimal combination of two genes (*SHP1*, *p15*) and of three genes (*SHP1*, *p15*, *p16*) of the analysed genes to predict this correlation. These data suggest that progression of the disease is associated with epigenetic deregulation of various regulatory pathways in CLL.

In summary, the *DLC1* gene is methylated in the majority of CLL patients. Patients with advanced Rai stage

at diagnosis tend to have simultaneously more methylated genes, suggesting that increasing methylation of tumour suppressor genes may contribute to disease evolution, or, at least, might reflect the molecular pathogenesis of CLL.

As the number of patients in our study was small, our observations must be validated in future prospective studies with larger numbers of patients.

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